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EVIDENCE THAT ULTRAVIOLET-INDUCED THYMINE DIMERS IN DNA CAUSE BIOLOGICAL DAMAGE

BY RICHARD B. SETLOW AND JANE K. SETLOW

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY*

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Ultraviolet radiation inactivates cells, viruses, and biologically active DNA's, destroys the priming activity of DNA in the calf thymus polymerase system,[1] and causes mutations. It is reasonable to suppose that much of the deleterious action of ultraviolet light arises from photochemical changes in DNA, such as chain breakage, cross-linking of strands, hydration of the pyrimidines, and formation of dimers between adjacent thymine residues in the polynucleotide chains (see refs. 2 and 3 for reviews). It has not been possible to show that any of these changes leads to biological inactivation, although many such interpretations have been made. A fundamental difficulty in identifying photochemical products with ultraviolet-induced biological damage is that increasing ultraviolet doses result in increasing numbers of all types of products, and those made in even small amounts may have devastating biological effects. If some of the photochemical events could be increased or decreased relative to others during biological inactivation, one could correlate biological alteration with relative amounts of photochemical products per DNA molecule.

The ratio of thymine dimers to other products in irradiated DNA can apparently be altered under appropriate conditions, and the present work gives evidence that ultraviolet-produced thymine dimers in *Hemophilus influenzae* transforming DNA lead to a loss in transforming activity. Thymine dimers are not, however, sufficient to explain all the observed inactivation by ultraviolet radiation.

Materials and Methods.—In the biological experiments, transforming DNA† from streptomycin- and cathomycin-resistant *H. influenzae* was suspended in 0.1 *M* NaCl at a concentration of 1 μg/ml and irradiated at room temperature. Meth-

ods of irradiation and transforming assay have been given previously.[4] Antibiotics were added to the top agar layer to give a final concentration of 5 μg/ml cathomycin and 500 μg/ml streptomycin. Absorbance changes due to ultraviolet irradiation were determined using the same preparation of DNA, but at a concentration of 30 μg/ml, in a Beckman spectrophotometer, with an unirradiated sample as a blank.

Experimental Results and Discussion.—Dimers, $\widehat{TT}$, between adjacent thymine residues, T, T, in polynucleotides are both made and broken by ultraviolet radiation,[5-7] but the action spectra for the two processes are different. Such dimers have been isolated from the DNA of irradiated bacteria.[8] The constants σ_f and σ_b, which represent cross sections with units cm^2/incident quantum, are wavelength-dependent and are *different* functions of wavelength.[5-7, 9] At any given wavelength a large dose of radiation yields a steady state in which the fraction of T,T sequences dimerized is $\sigma_f/(\sigma_f + \sigma_b)$. Because the σ's have different wavelength dependencies, the fraction of T,T sequences existing as dimers is also wavelength dependent. Similar photochemical changes are known for uracil,[11] but have not been found for DNA bases other than thymine.

The maximum possible number of dimers formed is less than the number of T,T sequences,[10] because groups such as T,T,T will only form one dimer, even though they represent two T,T sequences; furthermore, at very high doses other side reactions may occur before a dimer is formed, and in native DNA some T,T's may be held so rigidly in a double helix that the probability of forming a dimer is small.

Table 1 shows values of these constants for polythymidylic acid. It is apparent that at long wavelengths the equilibrium is well over on the side of dimers, but that shorter wavelength irradiation yields an equilibrium value of only 14 per cent dimers. Thus DNA heavily irradiated at 2800 A contains large numbers of dimers. If this DNA is subsequently exposed to radiation of shorter wavelength, dimers are broken and the new equilibrium value is quickly approached, as indicated by the large values of σ_b.

Two types of experiments suggest that thymine is implicated in biological alteration by ultraviolet radiation. First, a reactivating effect by 2390 A radiation has been shown for DNA priming activity in a calf thumus polymerase system.[1] The activity previously had been destroyed by 2800 A radiation. Second, the ultraviolet action spectrum for reversion of two T4 point mutants irradiated during the latent period resembles the thymidine absorption spectrum.[14] If thymine dimers are involved in biological damage, transforming DNA inactivated by 2800 A radiation should be partially reactivated by subsequent exposure to radiation of shorter wavelength.

The relation between transforming activity and incident ultraviolet dose is a complicated one, even though at 2800 A the fraction of T,T dimers[8, 13] and presumably other photochemical products increases almost linearly with dose. If we separate the photochemical changes into dimers and other unknown products, X, and let $(\widehat{TT})$ equal the fraction of T,T sequences which are dimers, we may represent the survival, S, as a function of these products by the equation

$$S = f\,[(X), (\widehat{TT})], \tag{1}$$

where both (X) and $(\widehat{TT})$ increase with dose, D. It has been found that, for small doses, at all wavelengths investigated,[4]

$$\sqrt{1/S} = 1 + \alpha_\lambda D \qquad (2)$$

and the sensitivity constant, α_λ, is wavelength dependent. At high doses this simple relation breaks down, as may be seen in Figure 1, and as was suggested previously.[15]

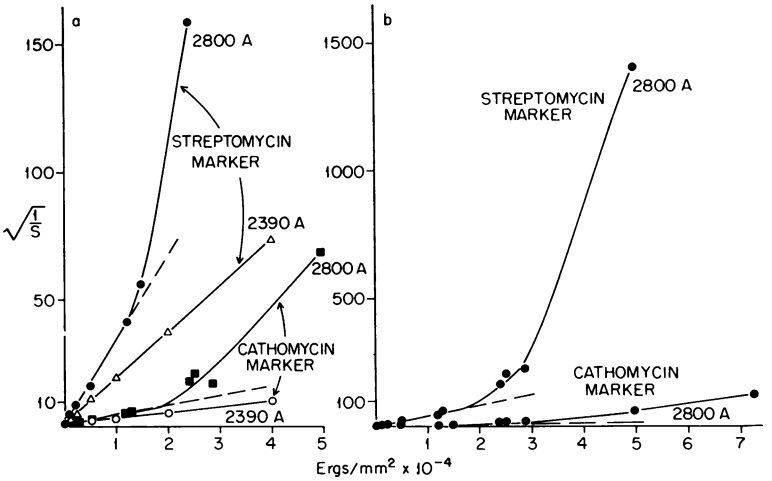

FIG. 1.—Inactivation of ability of irradiated DNA to transform. Note that the ordinate scales are different in a and b. S is the number of cells transformed with irradiated DNA divided by the number of cells transformed with unirradiated DNA. The dotted lines are extensions of the straight-line portions of the curves. There is a large error in the determination of the highest dose point at 2,800 A for the streptomycin marker in b, since only one transformed colony was observed.

The deviations from equation (2) depend on wavelength, since at 2800 A for the streptomycin marker the deviation occurs at a survival level which is in the straight-line portion on the 2390 A graph. This means that at high doses the function, f, of equation (1) is different for different wavelengths, and implies that at high doses the proportions of different inactivation processes differ with wavelength.[3]

The use of two normally inactivating wavelengths in sequence allows us to produce large variations in $(\widehat{TT})$ with only small variations in (X) in equation (1), and hence permits an evaluation of the relative roles of $\widehat{TT}$ and X in the inactivation process. This specific effect on $\widehat{TT}$, which has not been observed for other products, may be shown actually to occur by chemical isolation of dimers in irradiated DNA,[13] and is clearly seen in observations of the absorbance of irradiated native DNA, where formation of $\widehat{TT}$ causes a decrease in absorbance because the 4,5 double bond is saturated.[12] Photohydration[16] causes a similar decrease, but is a minor process in native DNA.[7] Figure 2 shows how the absorbance changes in DNA with various combinations of irradiation wavelengths. The decrease in absorbance with 2800 A radiation is reversed to a large extent by 2390 A radiation, to a smaller extent by 2480 A, and not at all by 2650 A. The pattern of absorbance changes, and by inference, $(\widehat{TT})$ changes, is similar to that given in Table 1 for

polythymidylic acid. For example, Figure 2 shows that, for DNA, 5×10^4 ergs/mm^2 at 2800 A followed by 2×10^4 ergs/mm^2 at 2390 A yields the same absorbance change as a single dose of around 1.5×10^4 ergs/mm^2 at 2800 A, a result in agreement with the value for polythymidylic acid (Table 1).

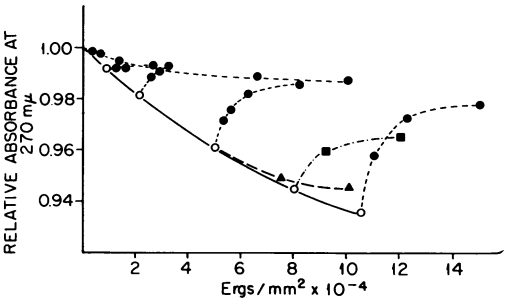

FIG. 2.—Absorbance changes in DNA resulting from various combinations of irradiating wavelengths. Where two wavelengths have been used in succession, the doses are plotted as the sum of doses at the two wavelengths. ○ 2,800 A, ● 2,390 A, ■ 2,480 A, and ▲ 2,650 A.

The survival of transforming activity following sequences of irradiation at 2800 A plus 2390 A is shown in Figure 3. *The changes are similar to those shown in Figure 2*, and are obviously different from the simple sum of inactivations at the individual wavelengths. Reactivation is observed if the initial dose of 2800 A is greater than 1×10^4 ergs/mm^2. The amount of reactivation by 2390 A radiation, relative to the survival before 2390 A, increases with increasing dose at 2800 A. The survival plateau that the reactivated samples reach decreases with increasing dose at 2800 A, indicating that processes other than T,T dimerization are also responsible for inactivation.

TABLE 1

AVERAGE CONSTANTS OF THE FORWARD AND BACK REACTIONS, T, T $\underset{\sigma b}{\overset{\sigma f}{\rightleftharpoons}}$ $\widehat{TT}$*

Wavelength (A)	σf (10^{-19} cm^2/quantum)	σb (10^{-19} cm^2/quantum)	Equilibrium fraction of dimers $\sigma f/(\sigma f + \sigma b)$	Dose of 2800 A alone to yield same fraction of dimers (10^4 ergs/mm^2)
2390	8	47	0.14	1.1
2480	12	25	0.32	2.6
2650	16	2.5	0.82	12
2800	7	0.16	0.98	∞

* These data for polythymidylic acid have been calculated in terms of incident doses of different wavelengths. Changes in T, T obtained from ultraviolet absorbance changes, calculated from the data of Deering and Setlow.[6]

Figure 4 shows that it is the sequence of 2390 A following 2800 A which causes reactivation, since 2800 A following 2390 A yields no such effect. In the latter case, the initial inactivation by 2800 A following 2390 A is greater than predicted for 2800 A alone at the same survival level, because of the large number of undimerized T,T sequences.

Figure 5 shows that 2800 A followed by 2480 A or by 2650 A results in survival patterns also similar to the absorbance changes shown in Figure 2. In addition, the data for 2480 A reactivation indicate that several processes are occurring, since small doses reactivate but larger doses lead to a decrease in activity.

Suppose that the formulation of equation (1) is correct and that 2390 A radiation has no reactivating effect on X. We may assess the relative importance of $\widehat{TT}$ and X in inactivation by comparing the survival curves for 2800 A irradiation alone with those for 2800 A plus subsequent 2390 A doses producing maximum reactivation

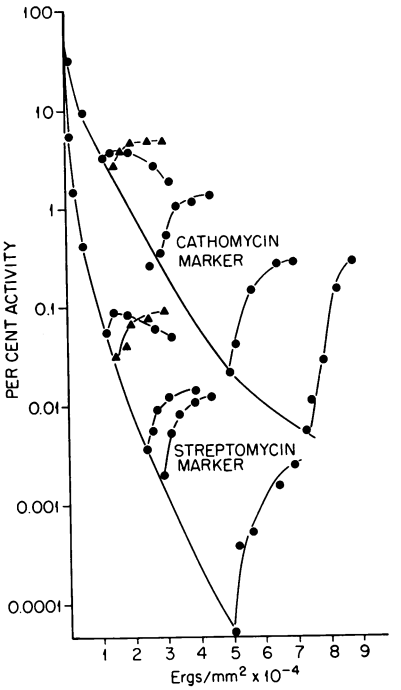

FIG. 3.—2,800 A irradiation followed by 2,390 A. The doses are plotted as the sum of the initial doses at 2,800 A and those at 2,390 A.

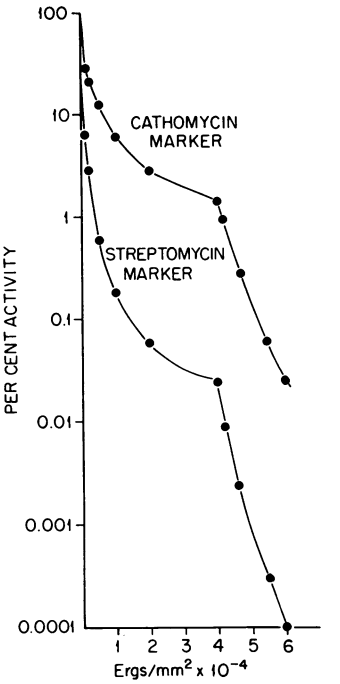

FIG. 4.—2,390 A irradiation followed by 2,800 A. Irradiation at 2,800 A was begun after a dose of 4×10^4 ergs/mm² of 2,390 A radiation, and doses beyond this point are given as the sum of this dose plus the increment of 2,800 A radiation.

(see Fig. 6). In the first case, both (X) and $(\widehat{TT})$ increase with dose at 2800 A, while, in the latter, for sufficiently high doses of 2800 A, $(\widehat{TT})$ is at a constant steady state value and only (X) increases with dose. The survival curves differ for doses above 1×10^4 ergs/mm², the dose of 2800 A which gives the maximum number of dimers obtainable with 2390 A (see Table 1). In the large dose range the survival curves are very roughly exponential, and the contributions of (X) and $(\widehat{TT})$ to inactivation may be found from the relative slopes of the survival curves. The negative of the slopes is proportional to the reciprocal of the additional dose necessary to decrease the activity by a factor, e. If $-k_1$ is the slope for 2800 A alone and $-k_2$ for 2800 A plus 2390 A, the relative contribution of $\widehat{TT}$ to inactivation is $(k_1 - k_2)/k_1$. This method of analysis yields, as seen in Table 2, the result that 50 per cent of the inactivation at high doses arises from the formation of thymine dimers.

Number of Thymine Dimers per Hit.—Using formal terms, we say that at high doses of 2800 A, $S \approx e^{-kD}$ and equation (1) becomes $\ln S = \ln f\,[(X), (\widehat{TT})] \cong -kD$. The slope of the survival curve is $d(\ln S)/dD \cong -k$. Since (X) and $(\widehat{TT})$ are functions of dose, D,

$$-k = \frac{\partial \ln S}{\partial(X)} \frac{\partial(X)}{\partial D_{2800\ A}} + \frac{\partial \ln S}{\partial(\widehat{TT})} \frac{\partial(\widehat{TT})}{\partial D_{2800\ A}} \equiv -k_X - k_{\widehat{TT}}. \quad (3)$$

TABLE 2

SURVIVAL CONSTANTS AT LARGE DOSES*

Marker	2800 A alone $-k_1$ (10^{-4} mm²/erg)	2800 A + 2390 A $-k_2$ (10^{-4} mm²/erg)	Relative contribution of $\widehat{TT}$ to inactivation $(k_1 - k_2)/k_1$
S	2.1	1.0	0.53
C	1.6	0.8	0.50

* The reciprocal of the dose of 2800 A necessary to reduce survival by a factor of e for doses greater than 1×10^4 ergs/mm² and less than 3×10^4 ergs/mm.²

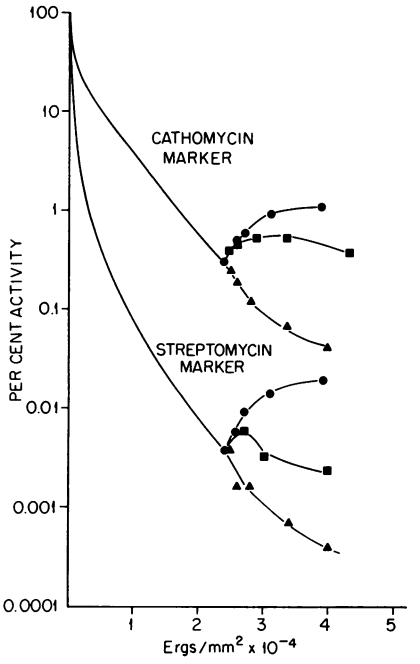

FIG. 5.—2,800 A irradiation followed by ● 2,390 A, ■ 2,480 A, and ▲ 2,650 A. The doses are plotted as the sum of the initial 2,800 A dose and the succeeding doses at 2,390 A, 2,480 A, or 2,650 A.

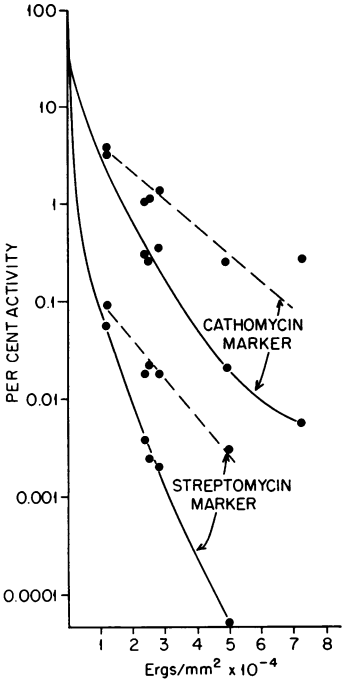

FIG. 6.— ——— 2,800 A alone. — — — 2,800 A plus optimal 2,390 A. The reactivation survival curves are the envelopes of the maximum survivals with 2,390 A radiation, obtained from data such as those of Figure 3. The doses shown are only the initial doses of 2,800 A.

For 2800 A irradiation alone $k = k_1 \equiv k_X + k_{\widehat{TT}}$.

For 2800 A followed by 2390 A, provided dose of 2800A is sufficiently large, $\partial(\widehat{TT})/\partial D_{2800\ A} = 0$, $k \equiv k_2 = k_X$. Hence,

$$k_{\widehat{TT}} = k_1 - k_2 \quad (4)$$

From the definition of $k_{\widehat{TT}}$ (equation 3)

$$\frac{\partial(\ln S)}{\partial(\widehat{TT})} = -\frac{k_{\widehat{TT}}}{\partial(\widehat{TT})/\partial D_{2800\text{ A}}} = -\frac{(k_1 - k_2)}{\partial(\widehat{TT})/\partial D_{2800\text{ A}}} \quad (5)$$

At 2800 A the $1/e$ dose for dimer formation in polythymidylic acid is 8.8×10^4 ergs/mm^2. Since this dose corresponds, on the average, to the formation of all possible dimers, $\partial(\widehat{TT})/\partial D_{2800\text{ A}} = 1/(8.8 \times 10^4)$, and the decrease in ln S for the formation of the maximum fraction of dimers $\Delta(\ln S)/\Delta(\widehat{TT})_{max}$ is found by substituting this number and those given in Table 2 into equation (5). For the cathomycin marker, $\Delta \ln S/1 = 0.8 \times 10^{-4} \times 8.8 \times 10^4 = 7$. A "hit" corresponds to $\Delta \ln S = 1$, so that in the high dose range the formation of the maximum number of dimers is the equivalent of 7 hits.

We get an independent estimate of this number from a comparison of the increase in survival and absorbance produced by 2390 A irradiation. Irradiation by 2390 A following 2800 A inactivation may be represented by a relation similar to equation 5 with the constants of the second member evaluated at 2390 A.

$$\frac{\partial(\ln S)}{\partial(\widehat{TT})} = -\frac{k_{\widehat{TT}}}{\partial(\widehat{TT})/\partial D_{2390\text{ A}}}. \quad (6)$$

Because for a large initial dose of 2800 A the right-hand side is constant, ln S is proportional to $(\widehat{TT})$, and ln S as a function of dose of 2390 A should be similar to the relative absorbance as a function of dose. Figure 7 shows a comparison of ln S versus D with the absorbance versus D. *The similarity of the two functions is quantitative evidence that breakage of thymine dimers restores biological activity.* The absorbance scale has been adjusted so that the maximum change in absorbance equals that in ln S. The absorbance changes at higher doses than those used in this work are only about one half those predicted from the known frequency of T,T sequences (around 10 per cent),[10] and are also one half of those observed in the irradiation of denatured DNA. Thus, irradiation of native DNA can form dimers in only about 5 per cent of the dinucleotide sequences. The formation of a dimer probably produces a local hyperchromic effect caused by electromagnetic uncoupling of adjacent bases that will compensate slightly for the loss in absorbance owing to dimer formation. As a result, dimerization of 5 per cent of the dinucleotides decreases the relative absorbance at 270 mμ by 0.08. A change in relative absorbance of 0.025 corresponds to a

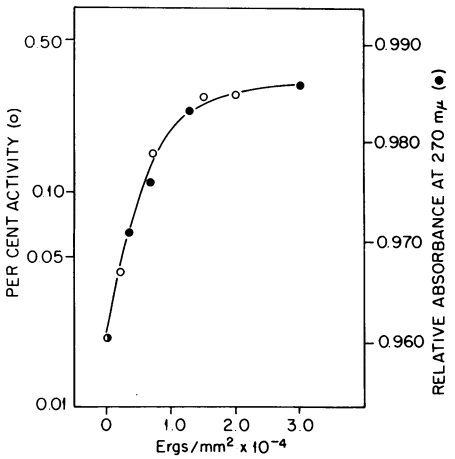

FIG. 7.—Correlation of change in absorbance with change in biological activity after irradiation with reactivating light. Data from Figures 2 and 3. Cathomycin marker irradiated at 2,390 A following 5×10^4 ergs/mm^2 at 2,800 A.

change in S of 15 times, or $\Delta \ln S$ of 2.7. The change 0.025 represents $\sim$0.3 of the maximum possible number of dimers and $\partial(\ln S)/\partial(\widehat{TT}) = 2.7/0.3 = 9$. The agreement between this number and the value of 7 obtained in a completely different way from the 2800 A inactivation data is excellent, and is further quantitative evidence that thymine dimers cause biological inactivation.

The change in ln S for the maximum possible number of dimers, about 8, means that an average of one "hit" per molecule (a change in ln $S = 1$) corresponds to $^1/_8$ of the maximum number of dimers. Since the maximum number is around 5 per cent of the dinucleotides or one dimer every 20 nucleotides (see preceding paragraph), an inactivating hit at large 2800 A doses corresponds to one dimer in every 160 nucleotides.

Summary.—The biological transforming activity of *Hemophilus influenzae* DNA inactivated by large doses of 2800 A radiation can be reactivated by subsequent irradiation at 2390 A. This reactivation has been correlated with a shift in the steady state distribution of the photochemical reaction causing adjacent thymine residues to form dimers. The kinetics and wavelength dependence for the biological reactivation are similar to those for the absorbance changes in irradiated DNA, which, in turn, are caused by making or breaking dimers. The data indicate that at high doses about 50 per cent of the biological inactivation is due to thymine dimer formation, and that one inactivating "hit" at high doses is the equivalent of the production of a dimer in every 160 nucleotides.

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